Dissecting pathways contributing to oncogenic transformation by the tyrosine kinase receptor TrkB

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CHAPTER 1

Epithelial-mesenchymal transition and anoikis resistance: two mechanisms promoting metastasis

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Metastasis is a multistep process in which tumor cells have to overcome multiple barriers in order to fully develop into a secondary tumor. One such step is the epithelial-mesenchymal transition, a process that enables the cells to invade surrounding tissues. Another process important for metastasis is anoikis resistance, which allows the cells to survive in the bloodstream. Both of these processes have been studied in the context of this thesis, and will be introduced in this chapter.

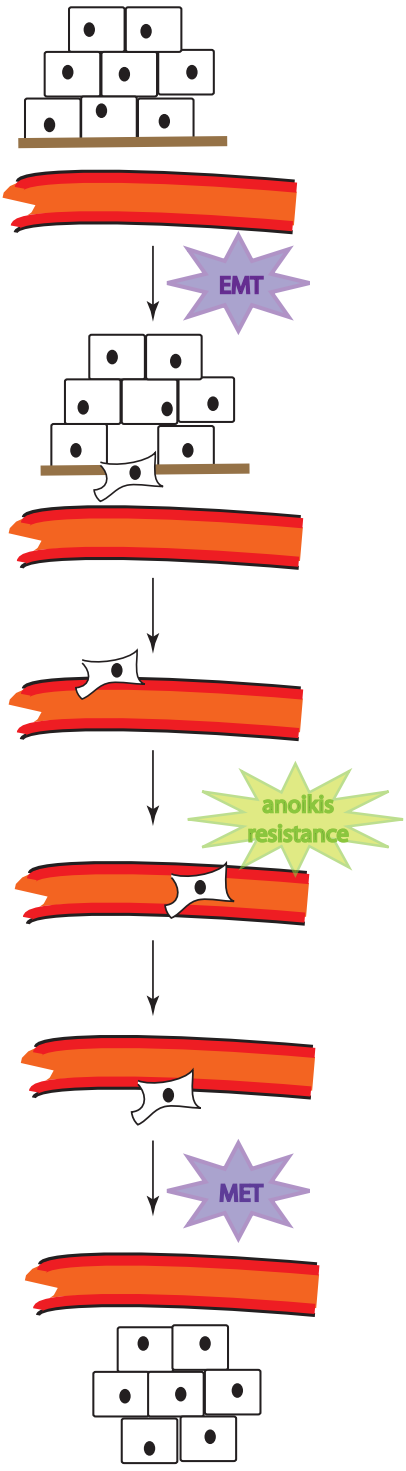
Metastasis

Most cancer patients die from metastases rather than primary tumors. Metastases undermine the function and structures of tissues and organs, and are generally difficult to combat. Understanding the underlying mechanism is therefore extremely important for the development of more successful therapies. Metastasis is a multistep process, in which the tumor cells have to cross many barriers in order to form a secondary tumor at a distant site (Fidler, 2003; Gupta & Massague, 2006). This process is very inefficient, because tumor cells must fulfill all steps of the metastatic cascade to achieve this. However, primary tumors can grow for several years before being detected, giving tumor cells ample opportunity to metastasize.

Metastasis initiates with epithelial tumor cells breaking through the basement membrane, leading to intravasation into the bloodstream or lymphatic vessels. Once arrived in the circulation, tumor cells must be able to survive in this unfamiliar environment, as they are often confronted with the loss of connections to the extracellular matrix (ECM) or neighboring cells. Next, at distant (organ) sites, they ought be able to adhere to the endothelium and extravasate from the bloodstream into the new host tissue. Finally, these so-called micro-metastases will need to have the capacity to grow out to macro-metastases or secondary tumors. Several processes play an important role in completing all of these distinct steps (Geiger & Peeper, 2009). Two of these will be discussed in detail below: epithelial-mesenchymal transition and anoikis.

Epithelial-mesenchymal transition

Once tumor cells start to invade their surrounding tissue, they are faced with an important barrier for metastasis: the basement membrane. One way to overcome this is by undergoing epithelial-mesenchymal transition (EMT) (Figure 1). This is a developmental process whereby epithelial cells lose their cell-cell contacts and acquire a more migratory phenotype. EMT is an important process for embryonic development and neural development (Thiery & Sleeman, 2006), but also during adulthood, for wound healing. It can also contribute to pathophysiological processes like renal fibrosis (Thiery et al., 2009).



Epithelial cells are connected to each other via so-called adherens junctions, tight junctions and desmosomes. Furthermore, they are attached to the ECM via focal adhesions. *In vitro*, EMT is characterized by the acquisition of a spindle-shaped morphology and scattering of the cells. The latter is achieved by disruption of adherens junctions. Upon EMT, epithelial markers, including E-cadherin and catenins (components of the adherens junctions), but also occludin and claudins (components of the tight junctions), are downregulated (Jechlinger et al., 2003). In addition to the downregulation of epithelial markers, EMT is often accompanied by the upregulation of mesenchymal markers. These include N-cadherin, vimentin, fibronectin and smooth muscle actin (Jechlinger et al., 2003). EMT does not always reflect a complete and unidirectional process: cells can also scatter reversibly by disassembly of the adherens junctions due to re-localization of specific molecules therein, without a change in the expression levels of the epithelial and mesenchymal markers (Grunert et al., 2003). This can be seen, for example, during development, when EMT is often followed by the reverse process (MET) (Thiery et al., 2009).

In vitro, EMT can be achieved by the over-expression of oncogenes, often in cooperation with transforming growth factor β (TGFβ). For example, (constitutively active) v-Src kinase or mutant Ras^{V12} plus TGFβ can

Figure 1: Distinct processes contributing to metastasis. Upon EMT, tumor cells can invade into the surrounding tissue and intravasate into the bloodstream. They are subsequently transported to distant sites of the organism. Once tumor cells lose their adhesive connections, they must be anoikis-resistant in order to survive at foreign locations. After extravasation from the bloodstream, tumor cells may undergo MET and form secondary tumors at distant sites.

induce EMT (Behrens et al., 1993; Janda et al., 2002). These oncogenes can activate several downstream pathways including the PI3K and the MAPK pathways. Activation of either can contribute to EMT (Grille et al., 2003; Lemieux et al., 2009). In fact, depending on the cell type and the activating oncogene, one or both pathways are required for EMT.

EMT in cancer

Several reports have described that EMT is important for cancer progression (Thiery, 2002; Christofori, 2006). In spite of this well-accepted idea among cancer biologists, pathologists usually have a hard time detecting EMT in tumors. Indeed, there is a longstanding debate about the role of EMT in metastasis (Tarin et al., 2005; Thompson et al., 2005). One reason why EMT is hard to detect *in vivo* could be the fact that it probably is a transient process. Secondly, only relatively few cells in the tumor invade into the bloodstream, which is likely to mask the detection of EMT. Thirdly, by the time tumor cells have formed a micro-metastasis, they may have undergone MET. Another reason why EMT is not easily detected in tumor tissue is proposed by an alternative model (Tsuji et al., 2009). This argues that cells that have undergone EMT and invaded into the bloodstream, allow cells that did not undergo EMT (the "non-EMT cells") to also get access to the circulation. Eventually, only the non-EMT cells will be able to form a secondary tumor (Tsuji et al., 2009).

Evidence in favor of a role for EMT in metastasis comes from the overexpression of several EMT regulators, which is commonly seen in cancer tissues. In most cases, this is correlated with disease progression (see below). In addition, in many cancer types, a typical cadherin switch is observed: the epithelial-specific E-cadherin is replaced by the more mesenchymal N-cadherin (Hazan et al., 2004). Along those lines, in prostate cancer, N-cadherin is expressed in the poorly differentiated areas, which are negative for E-cadherin (Tomita et al., 2000).

In spite of the disclaiming comments above, there are several reports that do show evidence of EMT taking place *in vivo*. For example, in spindle-shaped tumors of the mouse mammary gland, an EMT expression pattern was found (Damonte et al., 2007). In another study, a suicide gene under control of the fibroblast-specific protein-1 (FSP1) promoter was used. FSP1 is expressed only in fibroblasts, or epithelial cells that have undergone EMT. Because the suicide gene is under control of the FSP1 promoter, fibroblasts and "EMT-epithelial cells" are eliminated. When crossed with a polyomavirus middle T antigen (PyV-mT) breast cancer model, off-spring mice showed fewer metastases, showing that EMT is important for metastasis (Xue et al., 2003). Furthermore, several reports show by gene-expression profiling an EMT phenotype in carcinomas, correlating with high-grade carcinomas or metastasis (Alonso et al., 2007; Baumgart et al., 2007; Sarrio et al., 2008; Sheehan et al., 2008).

E-cadherin

The main component of adherens junctions is E-cadherin, a glycoprotein of the classical cadherin superfamily, comprising 26 members in humans. The structure of E-cadherin contains a large extracellular domain, a single transmembrane domain and a short intracellular domain. The extracellular domain consists of 5 similar subdomains (EC1-5), which are responsible for the formation of homophilic interactions with E-cadherin molecules of neighboring cells. This binding occurs in a zipper-like structure and is calcium-dependent (Takeichi, 1995). The cytoplasmic part of E-cadherin can bind both β-catenin and γ-catenin (plakoglobin). These catenins can bind α-catenin, which in turn can interact with the actin cytoskeleton. In this way, E-cadherin is linked to the cytoskeleton of cells, forming rigid cell-cell contacts (Perez-Moreno et al., 2003). Also another catenin, p120, can bind to E-cadherin in the cytoplasmic part (Shibamoto et al., 1995), thereby influencing the stability of the adherens junctions (Ishiyama et al., 2010).

E-cadherin is a central player in EMT: specific neutralizing antibodies prevent MDCK cells from forming cell junctions (Gumbiner et al., 1988). E-cadherin knockout mice are embryonic lethal; the embryos cannot normally develop into blastocytes, because they fail to form a mature epithelium (Larue et al., 1994). This supports the involvement of E-cadherin in embryogenesis and morphogenesis. Apart from these functions, E-cadherin also plays a role in tumorigenesis and metastasis (Cavallaro & Christofori, 2004) (see below).

Loss of cell-cell contacts or adhesion junctions alone is insufficient to drive metastasis. Expression of a dominant-negative truncated mutant of E-cadherin, which only titrates cytoplasmic proteins associated with the adherens junctions but does not affect downstream functions, induces cell scattering but has no effect on invasion of HMLER (HMLE cells overexpressing Ras^{V12}). However, silencing of E-cadherin in the same cells by shRNA results in regulation of downstream effectors and increased invasive and metastatic properties implying that E-cadherin can inhibit metastasis by affecting downstream effectors, including Twist (Onder et al., 2008).

E-cadherin in cancer

E-cadherin has been reported to act as a tumor suppressor gene (Christofori & Semb, 1999). This role is supported, for example, in melanoma in which E-cadherin is expressed to lower levels compared to normal melanocytes (Hsu et al., 1996). Along those lines, several studies have shown by immunohistochemical (IHC) analysis that E-cadherin is often expressed to lower levels in more undifferentiated cancers, including hepatocellular carcinoma (Shimoyama & Hirohashi, 1991), breast (Sommers et al., 1991; Oka et al., 1993), gastric (Oka et al., 1992), prostate (Umbas et al., 1994) and head and neck cancer (Schipper et al., 1994). Undifferentiated cancers often behave more aggressively. Consistent with this notion, in breast cancer, E-cadherin is lost in infiltrative lobular carcinoma (ILC), an aggressive type of breast cancer (Moll et al., 1993; Oka et al., 1993). Furthermore, in hepatocelullar

carcinoma, E-cadherin downregulation is correlated with a decreased cancer-free interval, decreased overall survival and increased clinical aggressiveness (Yang et al., 2009) and poor prognosis (Umbas et al., 1994).

The *in-vitro* evidence for a role of E-cadherin in invasion and tumor cell aggressiveness is substantial. For example, treatment of MDCK cells with an antibody against E-cadherin causes the cells to be more invasive into collagen and embryonic chick heart (Behrens et al., 1989). Along those lines, MDCK cells that overexpress Ras^{V12} show increased invasion into embryonic chick heart upon E-cadherin silencing (Vleminckx et al., 1991). Conversely, re-expression of E-cadherin in breast and bladder carcinoma cell lines renders the cells less invasive (Frixen et al., 1991).

Extending these observations to *in-vivo* relevance, overexpression of E-cadherin in MDA-MB-231 cells inhibits the number of bone metastases after intracardiac injection (Mbalaviele et al., 1996). More *in-vivo* evidence in favor of a role of E-cadherin in preventing metastasis came from mouse models. Homozygous loss of E-cadherin in combination with p53 loss in mammary epithelial cells induces ILC in mice (Derksen et al., 2006). Furthermore, crossing Rip1Tag2 mice, which express SV40 T antigen under control of the insulin promoter and develop carcinomas in the pancreas, with Rip1-E-cadherin mice leads to the development of dramatically fewer carcinomas, but more adenomas instead. Likewise, off-spring mice from the Rip1Tag2 mice and Rip1dnEcadherin (dominant negative form of E-cadherin), develop more carcinomas and form metastases in the pancreatic lymph node (Perl et al., 1998). This implies that loss of E-cadherin expression is critical for the switch from adenoma to carcinoma *in vivo*.

E-cadherin regulation

There are multiple ways in which E-cadherin function can get lost. One way is by hypermethylation. For example, in human breast and prostate carcinoma cell lines E-cadherin expression is often reduced by hypermethylation (Graff et al., 1995). Also in gastric cancer, one allele of E-cadherin is commonly inactivated by DNA hypermethylation (Grady & Peek, 2002). Another way of losing E-cadherin function

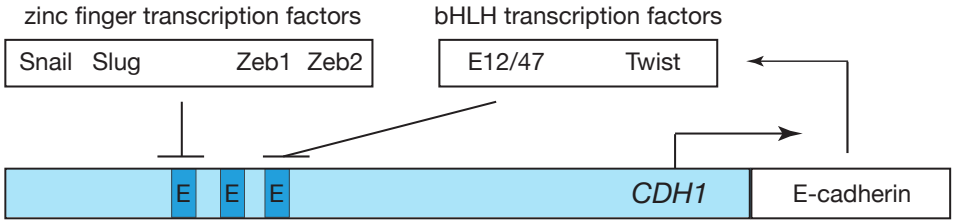


Figure 2: E-cadherin regulation by transcription factors. Transcription factors can bind to E-boxes in the *CDH1* promoter, thereby preventing its transcription. In turn, E-cadherin can induce Twist in a regulatory feedback loop.

is by mutation or loss of the complete locus. Mutations have been found in some cancer types, including ILC and gastric cancer (reviewed in (Berx et al., 1998)). For hereditary diffuse gastric cancer, these mutations account for 30-50% of the cases (Oliveira et al., 2009). A third way of losing E-cadherin function is by transcriptional downregulation. Several transcription factors can bind to E-boxes within the *CHD1* promoter and thereby suppress its transcription (Figure 2). These factors include E12/E47 (Perez-Moreno et al., 2001), Twist (Yang et al., 2004), Snail family and Zeb family members (Peinado et al., 2007). Recently, it has been found that miR-200b can regulate E-cadherin via the polycomb protein Suz12. Decreases in miR200b lead to enhanced binding of Suz12 to the *CDH1* promoter, thereby repressing E-cadherin transcription and resulting in enhanced growth of cancer stem cells (Iliopoulos et al., 2010).

Catenins

Catenins link E-cadherin to the actin cytoskeleton. However, β-catenin can also bind to transcription factors of the T-cell factor / lymphoid enhancing factor (TCF/LEF) family in the nucleus, thereby activating the Wnt signaling pathway and promoting cell migration (Willert & Nusse, 1998). β-catenin has been proposed to act as an oncogene, with a dual role in cell signaling: phosphorylation of β-catenin results in its rapid degradation (mediated by the APC protein) and disruption of the adherens junctions. If β-catenin cannot be degraded, more cytosolic β-catenin can translocate to the nucleus and activate the Wnt-signaling pathway, inducing several target genes including Myc (Willert & Nusse, 1998).

Also other catenins play a role in metastasis. For example, γ-catenin is associated with poor clinical outcome in oral squamous cell carcinoma (Narkio-Makela et al., 2009) and bladder cancer (Syrigos et al., 1998). For α-catenin, mutations have been reported in prostate cancer, which prevent it from binding to E-cadherin, thereby inhibiting cells from forming proper adherens junctions (Morton et al., 1993). Furthermore, upregulation of α-catenin is associated with lymph node metastasis in colorectal cancer (Elzagheid et al., 2008) and oral squamous cell carcinoma (Tanaka et al., 2003).

Twist

Twist proteins are basic helix loop helix (bHLH) transcription factors that can bind DNA through the consensus sequence CANNTG, called E-boxes. These consensus sites are found in the regulatory elements of many genes, including E-cadherin. Twist proteins have 2 family members: Twist1 (here referred to as Twist) and Twist2 (also known as Dermo-1). Twist1 and Twist2 are very similar in structure, with Twist2 lacking only a glycine rich motif (Li et al., 1995). Twist can be inhibited by Id proteins, which are similar in structure but lack the DNA binding site, thereby acting as dominant negatives (Pesce & Benezra, 1993). Twist knockout mice die before birth, presumably because Twist is required for cranial neural tube morphogenesis (Chen & Behringer, 1995).

As already mentioned, Twist can induce EMT by direct binding to the E-boxes in the *CDH1* promoter, thereby repressing E-cadherin transcription (Yang et al., 2004). However, binding of Twist to E-boxes does not always cause repression; it can also result in activation of transcription (Laursen et al., 2007), for example for some mesenchymal markers, like fibronectin (Yang et al., 2007b) and N-cadherin (Alexander et al., 2006). Besides regulating epithelial and mesenchymal markers, Twist can also bind to Bmi1, a member of the polycomb-repressive complex critical for the self-renewal potential of stem cells (Yang et al., 2010), thereby promoting the stem-cell-like properties of cells (Martin & Cano, 2010).

Twist in cancer

In breast cancer, Twist is found overexpressed in ILC (Yang et al., 2004). Furthermore, its expression is correlated with high-grade invasive breast carcinomas and chromosomal instability (Mironchik et al., 2005). Also in neuroblastoma, Twist expression correlates with aggressiveness, as well as with N-myc amplification (Valsesia-Wittmann et al., 2004). In this setting, Twist is thought to suppress N-Myc-induced apoptosis. Also in other settings, it protects against apoptosis, giving tumor cells a survival advantage (Maestro et al., 1999), for example to acquire resistance to chemotherapy (Pham et al., 2007). Through this property, Twist promotes the resistance to adriamycin (Li et al., 2009), taxol and vincristine (Wang et al., 2004; Zhang et al., 2007a).

Twist expression correlates with tumor stage in prostate (Kwok et al., 2007), ovarian (Yoshida et al., 2009) and bladder cancer (Zhang et al., 2007b). It is also associated with poor clinical outcome in colorectal cancer (Okada et al., 2010), hepatocellular carcinoma (Yang et al., 2009), cervical cancer (Shibata et al., 2008) and non-small cell lung cancer (NSCLC) (Hung et al., 2009). Twist expression correlates with myometrial invasion and overall survival of endometrial cancer patients and has therefore been proposed as a predictor of patient survival (Kyo et al., 2006). Since Twist expression is often correlated with aggressiveness and poor clinical outcome, it is easy to imagine that it plays an important role in metastasis. Indeed, Twist expression correlates with lymph node metastasis in bladder cancer (Zhang et al., 2007b), nasopharyngeal carcinoma (a head and neck tumor) (Horikawa et al., 2007) and esophageal squamous cell carcinoma (Sasaki et al., 2009). The group of Weinberg found that the highly metastatic mouse breast cancer cell line 4T1 is less metastatic upon Twist silencing, after subcutaneous injection into nude mice (Yang et al., 2004), showing that Twist is critical for metastasis, at least in this murine cell system. Besides its role in metastasis, Twist can also induce vascular mimicry in hepatocellular carcinoma (Sun et al., 2010).

Twist regulation

Twist can be induced by several oncogenes including Src (Cheng et al., 2008) and EGFR (Lo et al., 2007). This is mediated by several targets, for example Stat3, which in turn can repress Twist expression by direct binding to the *Twist* promoter (Cheng

et al., 2008). Another way of Twist induction occurs via the NFkB pathway. In turn, Twist can regulate NFkB via downregulation of cytokines, thereby creating a regulatory feedback loop (Sosic et al., 2003). A third way of Twist regulation is via the hypoxia regulators Hif-1α and Hif-2α. Twist is a direct target of both gene products (Gort et al., 2007; Yang et al., 2008). Consistent with this, in pancreatic cancer Twist is induced after exposure to hypoxia (Hotz et al., 2007). Hif1α promotes metastasis, in a Twist-dependent manner. Interestingly, co-expression of Twist, Snail and Hif1α predicts the worst prognosis in head and neck cancer patients (Yang et al., 2008) and NSCLC (Hung et al., 2009).

Snail

The Snail family is a group of highly related zinc-finger transcription factors, comprising Snai1 (Snail), Snai2 (Slug) and Snai3 (Smuc). Zinc-finger domains consist of two conserved cysteine and histidine residues and function as sequence-specific DNA binding motifs. Snail can bind to E-box motifs in DNA and thereby, like Twist, prevent E-cadherin transcription (Batlle et al., 2000; Cano et al., 2000).

Snail promotes EMT not only by downregulation of E-cadherin, but also by suppressing the expression of tight junction proteins, including claudins (Martinez-Estrada et al., 2006) and occludin (Ikenouchi et al., 2003), and the demosomal protein plakophilin and multiple cytokeratins, which form a cytoplasmic network of intermediate filaments (De Craene et al., 2005). The effect of Snail-induced invasion can be explained by effects on multiple downstream targets. For example, Snail induces MMPs, including MMP2 (Yokoyama et al., 2003) and MMP9 (Jorda et al., 2005). Furthermore, Snail induces myosin Va by binding to the E-boxes within its promoter. Myosin Va is involved in actin-myosin dynamics, thereby being a critical factor for cell motility (Lan et al., 2010).

Snail has an important role in embryogenesis by being critical for the formation of the neural crest and mesoderm (Leptin, 1991). Snail knockout mice die in gastrulation, because of incomplete EMT (Carver et al., 2001). Besides its role in development, Snail is also involved in pathogenesis, including renal fibrosis (Boutet et al., 2006) and wound healing (Franci et al., 2006). Likewise, Snail expression is found in activated fibroblasts that support wound healing in mice (Franci et al., 2006).

Snail in cancer

Snail is found overexpressed in many cancer types, including colorectal cancer (Roy et al., 2005), spindle cell carcinoma of the head and neck (Kojc et al., 2009), thyroid (Hardy et al., 2007), gastrointestinal (Rosivatz et al., 2006) and pancreatic cancer (Hotz et al., 2007). In some cancer types high Snail expression is associated with a reduced cancer-free interval or lower overall survival, for example in hepatocellular carcinoma (Yang et al., 2009), non-small cell lung cancer (Hung et al., 2009; Yanagawa et al., 2009), breast cancer (Elloul et al., 2005) and ovarian cancer (Blechschmidt et al., 2007; Yoshida et al., 2009). A positive correlation with metastatic disease is

found in esophageal squamous cell carcinoma (Usami et al., 2008), pancreatic (Yin et al., 2007) and head and neck cancer (Yang et al., 2007a). In infiltrative ductal carcinoma, Snail expression is associated with tumor dedifferentiation (Blanco et al., 2002) and lymph node metastasis (Blanco et al., 2002; Come et al., 2006). Along those lines, Snail expression promotes breast cancer recurrence while high Snail levels predict decreased relapse-free survival of breast cancer patients (Moody et al., 2005). High Snail expression is also observed in tumors from mesenchymal origin, sarcomas and fibrosarcomas, and in the stromal cells close to carcinomas. (Franci et al., 2006).

An important role for Snail in aggressive cancers is supported by several reports showing a contribution by Snail in invasion and metastasis both *in vitro* and *in vivo*. For example, Snail is required for the invasion of epithelial cells (Cano et al., 2000). Extending this finding to an *in-vivo* setting, silencing of Snail in breast cancer cells results in fewer and slower growing tumors after orthotopic injection. Interestingly, after surgical removal of these tumors, lymph node metastasis re-expressed Snail, showing that Snail is critical for metastasis (Olmeda et al., 2007b). Along those lines, silencing Snail in HaCa4 cells (Olmeda et al., 2008) and the highly metastatic ovarian cancer cells (Yin et al., 2007) results in a strong reduction in metastasis after orthotopic injection. Likewise, orthotopic injection of pancreatic cancer cells overexpressing Snail shows an increased number of metastases in the liver and the lymph node (Yin et al., 2007). Furthermore, HNSCC cells overexpressing NBS-1 (a metastasis promoter) were less metastatic upon Snail silencing by RNAi in an experimental metastasis model (Yang et al., 2007a).

All of the above suggest that Snail may serve as an attractive anticancer target. Attempts to target Snail in an anticancer therapy have been made, for example by viral application of anti-sense Snail to tumor cells inoculated into the mammary fat pad. This treatment resulted in a drop in the number of subaxillary lymph node metastases and increased survival of mice (Zhang et al., 2008). Furthermore, MIN mice (which as a result of a truncation in APC develop intestinal adenomas) treated with anti-sense Snail using PMO (phosphorodiamidate morpholino oligomer) show decreased tumorigenesis (Roy et al., 2004). Of note, these treatments have not been performed on established tumors.

Intratumoral injection with Snail siRNA decreases both tumor growth and the number of micro-metastases, but also increases the immune response (Kudo-Saito et al., 2009). In fact, Snail has been demonstrate to upregulate pro-inflammatory proteins including IL-6, IL-8 and CXCL1 (Lyons et al., 2008), suggesting that it could also promote cancer in a completely different way, by acting on the immune system. Another mechanism by which Snail can promote cancer progression is by its ability to protect cells against apoptosis (Vega et al., 2004). Lastly, Snail-overexpressing tumors results in increased angiogenesis (Yanagawa et al., 2009). Likewise, Snail has can induce the angiogenic markers CD31 and MECA32 (Peinado et al., 2004;

Olmeda et al., 2007a; Olmeda et al., 2007b; Olmeda et al., 2008), implying that it may promote angiogenesis. Therefore, Snail can promote cancer by several independent mechanisms.

Snail regulation
Snail can be regulated in different ways. Firstly, it can bind to its own promoter and thereby repress its own transcription, constituting a negative feedback loop (Peiro et al., 2006). Secondly, Snail can be regulated by phosphorylation. GSK3β can phosphorylate Snail at two sites, the first resulting in β-Trcp-mediated ubiquitination and proteasomal degradation and the second being responsible for subcellular localization of Snail (Zhou et al., 2004). This can be controlled by Wnt-1, which inhibits Snail phosphorylation, thereby stabilizing it and subsequently leading to invasion (Yook et al., 2005; Yook et al., 2006). Snail has also multiple upstream regulators, for example NFκB (Julien et al., 2007) and growth factors like HGF (Grotegut et al., 2006).

Zeb1
Zeb1 (or deltaEF1, TCF8) and Zeb2 (known as SIP1) form the Zeb transcription factor family. They are zinc finger transcription factors that are highly similar in structure, consisting of two clusters of zinc fingers and a homeodomain (van Grunsven et al., 2001). Zeb1 was first identified as a protein capable of binding to the chicken Δ crystalline enhancer (Funahashi et al., 1993) and later E-boxes of the immunoglobulin heavy chain (Genetta et al., 1994). Zeb1 knockout mice die soon after birth, because of severe skeletal defects (Takagi et al., 1998), showing that Zeb1 contributes to development of skeletal structures.

Zeb1 induces EMT by repressing E-cadherin transcription via binding to E-boxes in the corresponding promoter (Eger et al., 2005). Apart from E-cadherin, Zeb1 can repress additional genes, again by binding to multiple E-boxes in their promoters. The space between two adjacent E-box sites is critical for optimal repressing activity (Remacle et al., 1999). More downstream transcriptional targets include the polarity factor Lgl2 (Spaderna et al., 2008) and lama3α, a basement membrane marker (Spaderna et al., 2006). The latter may explain how Zeb1 contributes to the breakdown of the basement membrane, thereby stimulating invasion.

Zeb1 in cancer
Zeb1 is found overexpressed in several cancer types and is often correlated with aggressiveness. For example, in gall bladder cancer, Zeb1 expression is found in invasive tumors, specifically at the sites of cancer invasion (Adachi et al., 2009). Likewise, Zeb1 expression is found in high-grade lung adenocarcinoma (Dohadwala et al., 2006), leimyosarcomas (Spoelstra et al., 2006) and in aggressive endometrial cancers (Spoelstra et al., 2006; Singh et al., 2008). In colon cancer, high Zeb1 expression can be detected in polyps in surgical resections (Pena et al., 2005). Furthermore, high Zeb1 expression is found at tumor margins exhibiting tumor cell dedif-

ferentiation, showing that it is inversely correlated with cancer cell differentiation (Aigner et al., 2007). More evidence that Zeb1 is critical for metastasis comes from the observation that silencing Zeb1 in colorectal cancer cells reduces the number of metastatic lesions in the liver after intrasplenic injection in nude mice (Spaderna et al., 2008).

Zeb1 regulation
The miR-200 family, encoding several inducers of epithelial differentiation, can regulate Zeb1 expression (Gregory et al., 2008). More specifically, miR-200a, b, c, but also miR-141, can repress Zeb1 by binding to its promoter (Hurteau et al., 2007; Korpal et al., 2008; Park et al., 2008). This regulation is in a feed-forward loop, since Zeb1 can repress the miR-200 family as well (Bracken et al., 2008; Burk et al., 2008). In this way, tumor cells that have high Zeb1 levels have an efficient way of promoting metastasis. Furthermore, Zeb1-induced repression of the miR-200 family promotes tumorigenicity by inducing stemness (Wellner et al., 2009). Therefore, Zeb1 is not only important for metastasis, but also for tumor-initiating capacity (Brabletz & Brabletz, 2010).

Anoikis
Once tumor cells leave the primary site and start invading neighboring tissue or circulation, they are faced with another barrier: anoikis. Anoikis is a specific form of cell death, and is described as apoptosis caused by loss of cell adhesion. Tumor cells have to be able to survive in unfamiliar environments, like the bloodstream, and protect themselves against the “normal” response of healthy cells: anoikis. This process is carried out by several factors of the apoptotic machinery, including Bax, Bad, Bim, Bcl2 and caspases. It entails the activation of one of several pathways. For example, it can involve the release of cytochrome c (Grossmann et al., 2001) or the activation of the Fas pathway (Frisch & Screaton, 2001), dependent on the cell type. Anoikis plays an important role in tissue homeostasis and development, for example in mammary gland development (Talhouk et al., 1992; Boudreau et al., 1995).

Anoikis is most common for epithelial and endothelial cells (Meredith et al., 1993; Bates et al., 1994; Frisch & Francis, 1994). This notwithstanding, it has also been reported for fibroblasts (Frisch & Francis, 1994; McGill et al., 1997). However, there is little physiologic evidence for fibroblasts undergoing anoikis. Some early reports have shown that fibroblasts undergo anoikis when the adenovirus E1A oncoprotein is present (Frisch & Francis, 1994; McGill et al., 1997). One possible explanation for E1A to promote anoikis in fibroblasts is that it can activate effectors of the E2F pathway, of which some are apoptotic triggers.

Anoikis regulation
Cell-matrix interactions are mainly regulated by integrins, which are membrane-bound molecules that consist of two moieties: the α- and the β-subunit. Depending

on the cell type, specific integrins are responsible for the cell-matrix interactions. They are part of the focal adhesions and can signal to several molecules including ILK and focal adhesion kinase (FAK). In this way, integrins signal to several routes including the PI3 kinase and the MAPK pathway, both contributing to anoikis (Le Gall et al., 2000; Boisvert-Adamo & Aplin, 2006; Hehlgans et al., 2007). Several reports show different integrins to be critical for anoikis resistance (Frisch & Francis, 1994; Howlett et al., 1995). Furthermore, also other molecules of the focal adhesions play a role. For example, Talin-1, a focal adhesion protein that regulates integrin interaction with the ECM, protects epithelial cells from anoikis (Sakamoto et al., 2010). FAK, the main component of the focal adhesions, can also induce anoikis resistance (Frisch et al., 1996). Likewise, the integrin linked kinase (ILK), also part of the focal adhesions, can induce anoikis resistance (Attwell et al., 2000). Conversely, downregulation of FAK in tongue cancer cells increases anoikis sensitivity (Jiang et al., 2010).

Not only the cell-matrix interactions play a role in the regulation of anoikis; also cell-cell adhesion is important for protection against anoikis. Interestingly, keeping adherens junctions of colonic epithelial cells crypts intact before seeding them in suspension protects these cells from anoikis (Hofmann et al., 2007). The same was shown for proximal tubular cells seeded at a very high density on agarose plates (Bergin et al., 2000). Likewise, some adherens junction proteins have a role in anoikis protection. For example, N-cadherin promotes survival in melanoma and prostate cancer cells that are triggered to undergo anoikis (Li et al., 2001; Tran et al., 2002). Also β-catenin and E-cadherin can protect cells from going into anoikis (Kantak & Kramer, 1998; Orford et al., 1999; Kang et al., 2007; Ma et al., 2010). Other proteins, like Tropomyosin-1, a cytoskeletal protein and Rac1, a motility protein, can induce anoikis resistance, too (Coniglio et al., 2001; Bharadwaj et al., 2005).

Several oncogenes and tumor suppressors have been suggested to regulate anoikis. While anoikis can be mediated by the tumor suppressor p53 (Ilic et al., 1998), conversely, activated oncogenes like Src, Ras, BRaf, c-Met, EGFR, Erb2 and TrkB can induce anoikis resistance (Rak et al., 1995; McFall et al., 2001; Rosen et al., 2001; Windham et al., 2002; Zeng et al., 2002; Reginato et al., 2003; Douma et al., 2004; Boisvert-Adamo & Aplin, 2006; Haenssen et al., 2010). Several studies have used inhibitors of oncogenic kinases to induce anoikis *in vitro*. Examples are PP1 to inhibit Src kinase (Sakuma et al., 2010) and trastuzumab to inhibit HER2 (Pickl & Ries, 2009).

Anoikis in cancer

Anoikis resistance is a common characteristic of a tumor cell with metastatic potential. *In-vivo* evidence for anoikis playing a role in cancer progression came from a correlation between anoikis resistance and metastatic ability of oral squamous carcinoma cells: cells that are more anoikis-resistant show increased metastastatic ability (Swan et al., 2003). Also melanoma S91 cells that are selected for anoikis

resistance are more metastatic in nude mice (Zhu et al., 2001). In line with this notion, several rounds of anchorage depletion in melanocytes transforms them into more anoikis-resistant and cells displaying increased tumorigenic and metastatic potential (Oba-Shinjo et al., 2006).

The neurotrophic receptor TrkB

TrkB (Tropomyosin-related kinase B) or NTRK2 is a neurotrophic kinase receptor involved in neuronal survival. It is closely related to its family members TrkA (NTRK1) and TrkC (NTRK3). All Trk receptors respond to neurotrophin ligands: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). TrkB is mainly activated by BDNF and to lesser extent by NT-3 and NT-4. TrkB knockout mice are born alive, but die within hours, because they have lost both sensory and motor neurons, revealing the essential role of TrkB in neuronal survival (Klein et al., 1993). BDNF knockout mice have a slightly different and less dramatic phenotype: they show reduced growth and survival rates. Furthermore, they have sensory neural loss, although the motor neurons are intact (Ernfors et al., 1994; Jones et al., 1994).

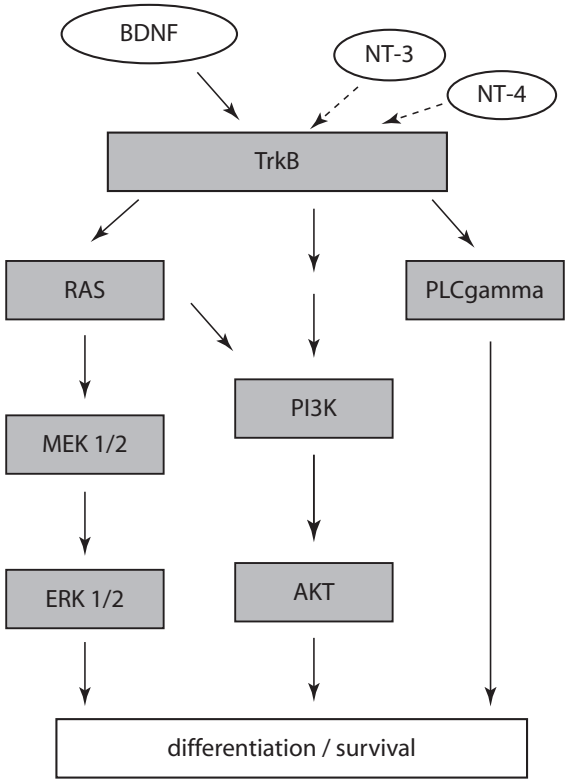


Figure 3: Pathways actived by TrkB. BDNF (and to a lesser extent NT-3 and NT-4) activates TrkB, leading to induction of several downstream signaling pathways, thereby regulating differentiation and survival.

The structure of TrkB consists of several extracellular domains, a single transmembrane domain and a cytoplasmic domain. Extracellular domains are responsible for the specificity towards the neurotrophin ligands. The cytoplasmic domain contains the kinase domain, which is essential for TrkB function, at least in rat epithelial model cell systems (Geiger & Peeper, 2007). Upon activation of TrkB by binding of the ligand, TrkB receptors dimerize and are phosphorylated on several tyrosine residues. In turn, active TrkB can activate multiple downstream path-

ways, including the PI3 and MAP kinase pathways and the PLCγ-pathway (Rubin & Segal, 2003) (Figure 3). TrkB signaling is also mediated via other kinases like Src (Huang & Reichardt, 2003) and c-Met (Hecht et al., 2005).

Consistent with the role of TrkB in anoikis resistance, TrkB expression renders non-transformed epithelial cells highly tumorigenic and metastatic (Douma et al., 2004). TrkB is found overexpressed in several cancer types, including Hodgkin lymphoma (Renne et al., 2005), prostate (Dionne et al., 1998), colon (Yu et al., 2010) and head and neck cancers (Zhu et al., 2007). Furthermore, TrkB is overexpressed preferentially in N-Myc-amplified neuroblastomas (Nakagawara et al., 1994), which is often correlated with poor prognosis (Brodeur, 2003). TrkB overexpression, is seen also other cancer types including Wilms' tumor (Eggert et al., 2001) and pancreatic cancer (Sclabas et al., 2005). In the latter, it is correlated with liver tumor recurrence. Along those lines, TrkB overexpression is found in high-grade ovarian carcinomas, in which it correlates with decreased survival of ovarian cancer patients (Yu et al., 2008; Au et al., 2009).

Considering the high levels of TrkB expression commonly seen in aggressive cancers, TrkB could be a suitable target for anticancer therapy (Ruggeri et al., 1999; Geiger & Peeper, 2005; Desmet & Peeper, 2006). Providing some support for this premise, an antibody cocktail against neurotrophins decreases tumor growth in mouse tumor xenografts (Miknyoczki et al., 2002). To establish new therapeutical agents, several Trk inhibitors are being developed, of which most target all Trk kinases. For example, the Trk inhibitor K252a induces apoptosis and decreases growth in soft agar in lung adenocarcinoma cells *in vitro* (Perez-Pinera et al., 2007). Derivatives of K252a, CEP-701 and CEP751, show an effect on tumor growth in several xenograft studies, including neuroblastoma (Evans et al., 1999; Iyer et al., 2010), pancreatic (Miknyoczki et al., 1999) and prostate cancer (Dionne et al., 1998; Weeraratna et al., 2001). In those settings, these drugs reduce the metastatic capability of prostate cancer cells that are subcutaneously injected into mice (Weeraratna et al., 2001). Although these drugs are not specific for TrkB, they had no effect on normal prostate gland growth (Dionne et al., 1998), showing at least some degree of specificity. Although phase I clinical studies with these inhibitors failed to show any severe toxicity (Undevia et al., 2004; Collins et al., 2007), the clinical effect remains to be optimized. Recently, two additional Trk inhibitors have been developed. The first, AZ-23, shows an inhibitory effect on TrkA-driven xenograft tumors *in vivo* (Thress et al., 2009). The second, cyclotraxin, a specific inhibitor for TrkB, shows antianxiety effects in mice (Cazorla et al., 2010). However, clinical effects of these inhibitors remain to be evaluated in cancer patients.

In conclusion, the metastasic capacity of tumor cells is built on several acquired properties, including the ability to undergo EMT and become anoikis resistance. Both processes involve a number of regulators, at least some of which could be potential drug targets. However, as most of those factors identified thus far are

transcription factors, which are difficult to target, future studies will be required to present us with novel entry points for therapeutic intervention.

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